ELSEVIER

Journal of Chromatography B, 692 (1997) 413–418

JOURNAL OF CHROMATOGRAPHY B

Determination of the halothane metabolites trifluoroacetic acid and bromide in plasma and urine by ion chromatography

Douglas C. Hankins[a], Evan D. Kharasch[a,b,c,*]

[a]*Department of Anesthesiology, University of Washington, Seattle, WA 98195, USA*
[b]*Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195, USA*
[c]*Anesthesiology Service, Puget Sound Veterans Affairs Medical Center, Seattle, WA 98108, USA*

Received 1 August 1996; revised 12 November 1996; accepted 18 November 1996

Abstract

Halothane ($CF_3CHClBr$), a widely used volatile anesthetic, undergoes extensive biotransformation in humans. Oxidative halothane metabolism yields the stable metabolites trifluoroacetic acid and bromide which can be detected in plasma and urine. To date, analytical methodologies have either required extensive sample preparation, or two separate analytical procedures to determine plasma and urine concentrations of these analytes. A rapid and sensitive method utilizing high-performance liquid chromatography–ion chromatography (HPLC–IC) with suppressed conductivity detection was developed for the simultaneous detection of both trifluoroacetic acid and bromide in plasma and urine. Sample preparation required only ultrafiltration. Standard curves were linear ($r^2 \geq 0.99$) from 10 to 250 μM trifluoroacetic acid and 2 to 5000 μM bromide in plasma and 10 to 250 μM trifluoroacetic acid and 2 to 50 μM bromide in urine. The assay was applied to quantification of trifluoroacetic acid and bromide in plasma and urine of a patient undergoing halothane anesthesia.

Keywords: Halothane; Trifluoroacetic acid; Bromide

1. Introduction

Halothane ($CF_3CHClBr$) is a widely used volatile anesthetic and the premier anesthetic agent for children. Halothane causes a rare but often fatal fulminant hepatic necrosis ("halothane hepatitis") resulting ultimately from oxidative halothane metabolism [1]. Halothane also elicits a more common yet milder form of toxicity, of lesser clinical consequence, that is caused by reductive halothane metabolism. Thus halothane toxicities are clearly related to halothane metabolism.

Halothane undergoes extensive biotransformation in humans, with approximately 50% of an absorbed dose metabolized [2]. Both oxidative and reductive pathways of halothane metabolism are evident during routine anesthesia [1]. Halothane is oxidatively metabolized to an unstable trifluoroacetyl halide with concomitant release of bromide. This halide intermediate reacts with water to produce trifluoroacetic acid (TFA) or acylates tissue molecules to form TFA-protein adducts. The major stable products of halothane oxidation are TFA, bromide and TFA adducts; thus plasma and urine TFA concentrations serve as reliable clinical markers for oxidative

*Corresponding author. Address for correspondence: Department of Anesthesiology, Box 356540, University of Washington, Seattle, WA 98195, USA.

PII S0378-4347(96)00527-0

halothane metabolism and TFA-adduct formation. Reductive halothane metabolism generates a free radical that can eliminate bromide to form 2-chloro-1,1,1-trifluoroethane (CTE), initiate lipid peroxidation or covalently bind to phospholipids and proteins, or undergo a second reduction to another free radical than eliminates fluoride to produce 2-chloro-1,1-difluoroethylene (CDE). The stable volatile products of halothane reduction are CTE and CDE and the stable soluble metabolites are fluoride and bromide. Thus plasma and urinary fluoride concentrations are clinical markers for reductive halothane metabolism. Plasma and urine bromide concentrations reflect both oxidative and reductive halothane metabolism.

Quantification of the stable halothane metabolites TFA, bromide and fluoride has been of considerable interest. Methods for the analysis of TFA have included gas chromatography with flame ionization detection [3–7], gas chromatography–mass spectrometry [8], fluorine nuclear magnetic resonance (^{19}F-NMR) [9], capillary isotachophoresis [10] and HPLC–ion chromatography (HPLC–IC) [11]. Many gas chromatographic methods are based on analysis of volatile TFA and bromide methyl esters, requiring extraction and/or derivatization schemes and special headspace autosamplers. The method of Maiorino et al. [6] utilized simple metabolite methylation without extraction, but was said to lack sensitivity [8]. Mass spectrometry was used to improve sensitivity, but partitioning of the methyl esters was extremely susceptible to changes in aqueous phase composition [8]. While gas chromatographic methods have the advantage of analyzing both TFA and bromide, the acidic conditions used to volatilize the derivatized metabolites are disadvantageous to chromatography columns and hardware. Liquid chromatographic techniques have the advantage of simpler sample preparation, however these methods have been applied to the exclusive analysis of TFA, rather than TFA and bromide [10,11]. Separate bromide analysis, traditionally by ion-selective electrode, is confounded by the high concentrations of chloride found in plasma and urine which interfere with electrode function, particularly at low bromide concentrations. Attempts have been made to compensate for chloride interferences with bromide electrodes [9].

Because of these current analytical limitations, we sought a new method for analysis of TFA and bromide in plasma and urine, applicable to pharmacokinetic studies of halothane metabolism in vivo. We describe a rapid and sensitive method for the simultaneous determination of bromide and TFA in plasma and urine by HPLC–IC using a simple sample preparation technique.

2. Experimental

2.1. Chemicals

Trifluoroacetic acid, sodium bromide, and dibromoacetic acid were purchased from Fluka Chemicals (Ronkonkoma, New York, USA). Sodium hydroxide (50%, w/w) low in carbonate was obtained from Fisher Scientific (Pittsburgh, PA, USA). Bovine and human serum albumin were purchased from Sigma (St. Louis, MO, USA). All water (>18 MΩ cm) was obtained from a Milli-Q UV plus ultrapure water system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Analyses were performed with a DX-300 HPLC–IC system (Dionex, Sunnyvale, CA, USA) consisting of an AGP gradient pump, LCM-3 chromatography module, CDM-3 conductivity detector, ASM-3 autosampler, ATC-1 anion trap column and an ASRS-1 anion self-regenerating suppressor operating in the autosuppression recycle mode, using a Dionex IonPac AS11 analytical column (4×250 mm) and AG11 guard column (4×50 mm). The detector sampling rate was 0.2 s. Dionex AI-450 software was used for hardware control, detector signal acquisition and chromatographic peak integration.

2.3. Operating parameters

Injections (50 μl) were made via the autosampler utilizing 0.5 ml polypropylene vials equipped with 20 μm filter caps (PolyVial, Dionex). For plasma analysis, the sodium hydroxide gradient profile was initially 0.5 m*M* for 5 min, linearly increased to 3.0 m*M* over 10.5 min, increased to 80 m*M* at 25 m*M*/min, and held at 80 m*M* for 5 min to elute any strongly retained anions. The concentration was linearly decreased to 0.5 m*M* at 14.5 m*M*/min, and

the column was allowed to reequilibrate at this concentration for 7 min.

For urine samples, the sodium hydroxide gradient was initially 0.5 m*M* for 5 min, linearly increased to 0.85 m*M* over 10 min, linearly increased to 3 m*M* over 5 min, then increased linearly to 76 m*M* over the next 3 min, where it remained for 5 min. The concentration was then decreased linearly to 0.5 m*M* over the next 4 min, and the column was allowed to re-equilibrate at this sodium hydroxide concentration for 10 min. The eluent flow-rate was 2.0 ml/min.

In an effort to prevent column performance degradation a column cleanup routine was performed every other day. The anion trap column was removed, the guard column was moved down-stream of the analytical column, and the eluent from the guard column was routed directly to a waste container. The sodium hydroxide gradient began at 0.5 m*M* and was linearly increased to 1 *M* over 15 min. The hydroxide concentration remained at 1 *M* for 60 min, and then linearly decreased to 0.5 m*M* over the next 15 min, where it remained for 20 min to allow the column to return to operational equilibrium. The eluent flow-rate was 2.0 ml/min throughout the cleanup.

2.4. Sample preparation

Plasma samples were thawed, and a 450 μl aliquot combined with 50 μl of the internal standard dibromoacetic acid (1 m*M*). Plasma was deproteinated by ultrafiltration using a Centrifree micropartition system with a 30 000 Da molecular mass cut-off YMT membrane (Amicon, Beverly, MA, USA). The ultrafiltration cartridge was first washed thoroughly with deionized water, filled with plasma, and then centrifuged for 60 min at 1700 *g*. The resulting ultrafiltrate was diluted with an equal volume of deionized water to provide an adequate volume for autosampler operation and transferred to autosampler vials for analysis. To compensate for endogenous bromide in patient plasma, the peak area ratio (bromide/dibromoacetic acid) from the initial (pre-anesthetic) sample was subtracted from all subsequent samples for that patient in order to quantify halothane-dependent bromide formation.

Frozen urine was thawed, and a 150 μl aliquot combined with 150 μl of the internal standard dibromoacetic acid (1 m*M*). This solution was filtered through a 0.22 μm membrane (Micropure-0.22 μm Separator, Amicon) by centrifugation at 16 000 *g* for 30 min. The resulting filtrate was diluted 10-fold with deionized water and transferred to autosampler vials. As with the plasma samples, endogenous bromide peak area ratios found in the pre-anesthetic sample were subtracted from all subsequent samples for that patient.

2.5. Calibration and quantification

Calibration standards were prepared daily using 450 μl outdated human plasma, 500 μl aqueous TFA and bromide standards, and 50 μl dibromoacetic acid. Final concentrations were 0, 10, 25, 50, 100 or 250 μ*M* TFA and 0, 2, 5, 10, 25, 50, 100, 500, 1000 or 5000 μ*M* bromide. These samples were treated identically to the patient samples, with the exception that they were not diluted prior to HPLC analysis. To compensate for endogenous bromide in the outdated plasma, bromide peak area ratios from samples containing 450 μl plasma, 50 μl internal standard and 500 μl deionized water were subtracted from those of subsequent calibration standards. Calibration standards for analysis of urine were prepared daily using saline, and were treated identically to the urine samples. Final concentrations were 0, 10, 25, 50, 100 or 250 μ*M* TFA and 0, 2, 5, 10, 25, or 50 μ*M* bromide. Saline was used to most closely approximate the concentration of chloride found in the experimental samples. Standard curves of peak area ratio (TFA/dibromoacetic acid and bromide/dibromoacetic acid) vs. analyte concentration were prepared and used to calculate analyte concentrations in patient samples. The resulting concentrations were multiplied by the appropriate dilution factor.

Quality control samples were prepared using blank urine and plasma at final concentrations of 10 μ*M* TFA and 2 μ*M* bromide. These were divided into 1 ml aliquots and frozen. Individual samples were thawed and analyzed daily with analytical samples.

2.6. Extraction recovery

Recoveries of TFA and bromide from plasma and urine were assessed. TFA and sodium bromide were added to outdated human plasma to achieve final

concentrations of 100 and 25 μ*M*, respectively. TFA and bromide were added to human urine to achieve final concentrations of 25 μ*M* each. To determine the influence of serum protein concentration on analyte recovery, TFA (25 μ*M* final), sodium bromide (25 μ*M* final), and bovine or human serum albumin (0–7 g/dl) were added to deionized water. Peak areas were compared to those of samples containing TFA and bromide in deionized water. Percent recovery was defined as $(C_{sample}/C_{std})\cdot 100$, where C_{sample} is the sample concentration in plasma, albumin solution, or urine, and C_{std} is the mean analytical standard concentration. Results are expressed as the mean of duplicates.

2.7. Analysis

Results are presented as the mean±standard deviation. Equations for the standard curves were analyzed by linear regression analysis, and parameters are reported ±the standard error of the estimate.

3. Results and discussion

Typical chromatograms for TFA, bromide and the internal standard dibromoacetic acid in plasma are shown in Fig. 1A Fig. 1B. A chromatogram of blank plasma, containing only dibromoacetic acid, is shown in Fig. 1A. A small bromide peak (1.6±0.9 μ*M*) was routinely observed in blank plasma, due to endogenous circulating bromide concentrations consistent with previous observations [12]. Standard curves were corrected to adjust for bromide present in the outdated human plasma. No other endogenous interfering peaks were observed in the region in which TFA, bromide and the internal standard eluted. A chromatogram of plasma obtained from a patient anesthetized with halothane is shown in Fig. 1B. TFA was adequately resolved from the chloride peak, but baseline resolution was not achieved in some samples. Nevertheless, peak integration and analyte quantification were readily accomplished.

Standard curves of peak area ratios, (TFA/dibromoacetic acid) and (bromide/dibromoacetic acid), versus analyte concentration in plasma were linear for 10–250 μ*M* TFA ($y=0.012\pm0.00017\cdot$[TFA]-0.070 ± 0.021, $r^2=0.999$) and 2–1000 μ*M*

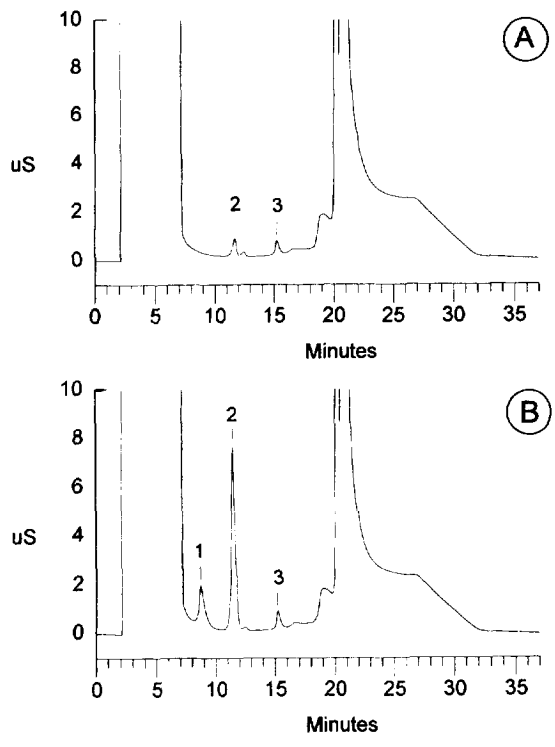

Fig. 1. Ion HPLC analysis of TFA and bromide in plasma. (A) Blank plasma containing the internal standard dibromoacetic acid (3). The large peak at 5 min is endogenous chloride. Small amounts of bromide (2) are naturally occurring. (B) Plasma obtained from a patient anesthetized with halothane. Retention times for TFA (1), bromide (2) and dibromoacetic acid (3) are 8.8, 11.5 and 15.5 min, respectively.

bromide ($y=0.017\pm0.0043\cdot$[Br]-0.037 ± 0.017, $r^2=1.00$). Inclusion of an additional calibration sample at 5000 μ*M* bromide also yielded a linear standard curve whose slope and intercept were statistically indistinguishable ($p=0.2$) from those of the smaller range standard curve, which was used for routine analysis. The limits of quantification, defined as the lowest point on the standard curve, were 10 μ*M* TFA and 2 μ*M* bromide. Observed concentrations of the plasma quality control samples at these concentrations were 10.1±2.4 μ*M* for 10 μ*M* TFA, and 2.4±0.4 μ*M* for 2 μ*M* bromide ($n=16$). Inter-day coefficients of variation for 100 μ*M* TFA were 4% (99.3±3.7 μ*M*) and 3% for 100 μ*M* bromide (98.1±2.6 μ*M*) ($n=6$). No attempts were made to increase assay sensitivity, which was sufficient for the pharmacokinetic studies.

Recovery of TFA from human plasma was found to be 72±8%, while bromide and the internal standard were essentially recovered quantitatively. Previous investigations have shown that TFA binds noncovalently to liver microsomal proteins, decreasing extraction efficiency [4]. Similar binding to plasma proteins could explain the incomplete TFA recovery we observed. There was also a concern that variability in plasma protein concentrations, both between patients as well as between patient plasma and the blank plasma used to prepare the calibration standards, may affect the accuracy of the results obtained. Therefore the influence of varying serum albumin concentrations on the recovery of TFA was determined, using both bovine and human serum albumin. Recovery of TFA was not significantly influenced by varying albumin concentrations over the range (2–6 g/dl). Since TFA recovery was similar throughout the range of normal clinical serum albumin concentrations (3–5 g/dl), minor differences in albumin concentration between patient plasma and that used for standard curves would not be expected to significantly affect the results.

Typical chromatograms for TFA, bromide and the internal standard dibromoacetic acid in urine are shown in Fig. 2A Fig. 2B. A chromatogram of blank urine, containing only dibromoacetic acid, is shown in Fig. 2A. Blank urine samples did not contain TFA, but did contain small amounts of endogenous bromide. No other endogenous interfering peaks were observed in the elution region for TFA, bromide and the internal standard. A chromatogram of urine obtained from a patient anesthetized with halothane is shown in Fig. 2B.

Standard curves of peak area ratios (TFA/dibromoacetic acid and bromide/dibromoacetic acid) versus analyte concentration in saline were linear for 10–250 μM TFA ($y=0.020\pm0.00049\cdot[\mathrm{TFA}]+0.075\pm0.060$, $r^2=0.998$) and 2–50 μM bromide ($y=0.035\pm0.00064\cdot[\mathrm{Br}]+0.019\pm0.016$, $r^2=0.999$).

Limits of quantification were 5 μM bromide (5.0±0.3, $n=4$) and 25 μM TFA (25.6±0.5, $n=4$). Metabolite recovery from urine was essentially complete, since no differences were observed between urine and saline samples containing 25 μM TFA and bromide.

The assay was applied to the quantification of TFA and bromide in the plasma and urine of a patient anesthetized with halothane. Typical plasma and urine concentrations in a patient anesthetized with 1% halothane for 3 h are shown in Fig. 3.

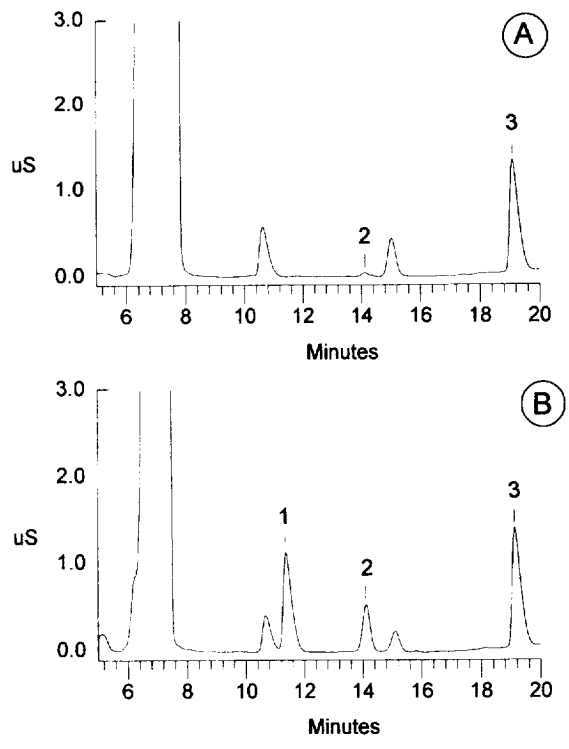

Fig. 2. Ion HPLC analysis of TFA and bromide in urine. (A) Blank urine from a subject not exposed to halothane which contained the internal standard dibromoacetic acid (3). (B) Urine from a patient anesthetized with halothane. Retention times for TFA (1), bromide (2) and dibromoacetic acid (3) are 11.2, 14.1 and 19.2 min, respectively. The large peak at 7 min is chloride.

Previous attempts to utilize ion chromatography with an SAX-1 column using a sodium carbonate–bicarbonate isocratic eluent system to measure TFA in the presence of physiologic chloride concentrations (150 mM) resulted in suboptimal chromatographic resolution of the relatively small TFA peak from the large chloride peak [11]. Attempts in our laboratory to ameliorate this problem by performing sample cleanup through silver-impregnated resin cartridges to remove chloride prior to HPLC analysis were not feasible because these methods also remove bromide. Rather, we obtained adequate resolution by utilizing an AS11 column with a hydroxide gradient elution.

The present assay, using hydroxide gradient elu-

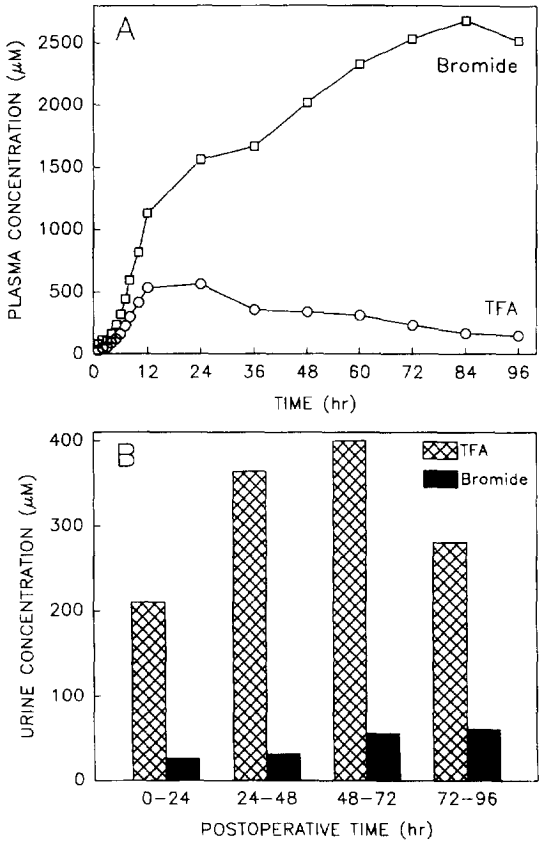

Fig. 3. Time-course of TFA and bromide concentrations in plasma and urine after halothane anesthesia. The patient received 1% halothane for 3 h.

tion HPLC-ion chromatography represents an improvement over existing HPLC assays which analyze TFA exclusively, and an alternative or potential improvement over existing gas chromatography–mass spectrometry or gas chromatography–flame ionization techniques which require extensive sample preparation. This assay uses a simplified sample preparation method and permits the simultaneous and sensitive analysis of both TFA and bromide.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01 GM48712) and by a Pharmaceutical Research and Manufacturers of America Foundation Faculty Development Award.

References

[1] D.C. Ray and G.B. Drummond, Br. J. Anaesth., 67 (1991) 84.
[2] R.L. Carpenter, E.I. Eger II, B.H. Johnson, J.D. Unadkat and L.B. Sheiner, Anesthesiology, 65 (1986) 201.
[3] D. Karashima, S. Takahashi, A. Shigematsu, T. Furukawa, T. Kuhara and I. Matsumoto, Biomed. Mass Spectrom., 3 (1976) 41.
[4] D. Karashima, A. Shigematsu, T. Furukawa, T. Nagayoshi and I. Matsumoto, J. Chromatogr., 130 (1977) 77.
[5] L. Witte, H. Nau, J.-H. Fuhrhop, A. Doenicke and B. Grote, J. Chromatogr., 143 (1977) 329.
[6] R.M. Maiorino, A.J. Gandolfi and I.G. Sipes, J. Anal. Toxicol., 4 (1980) 250.
[7] H. Wark, J. Earl, D.D. Chau and J. Overton, Br. J. Anaesth., 64 (1990) 474.
[8] L.D. Gruenke and L.A. Waskell, Biomed. Environ. Mass Spectrom., 17 (1988) 471.
[9] M.J. Cousins, J.H. Sharp, G.K. Gourlay, J.F. Adams, W.D. Haynes and R. Whitehead, Anaesth. Int. Care, 7 (1979) 9.
[10] M. Morio, K. Fujii, R. Takiyama, F. Chikasue, H. Kikuchi and L. Ribaric, Anesthesiology, 53 (1980) 56.
[11] R. Kawaguchi, K. Fujii, M. Morio, O. Yuge and D. Hossain, Hiroshima J. Med. Sci., 38 (1989) 27.
[12] F.M. Corrigan, E.R. Skinner, F. MacIntyre, G. Ijomah, J. Holliday, D.F. Horrobin and N.I. Ward, Clin. Chim. Acta, 204 (1991) 301.